NASA TECHNICAL TRANSLATION

NASA TT F-13,652

INVESTIGATIONS INTO THE GROWTH OF *BACILLUS COLI* BY DIRECT MICROSCOPIC OBSERVATION. THE PERIODICITY OF BACTERIOPHAGE ACTIVITY STUDIED ON THE BASIS OF THE OBSERVATIONS MADE IN THE COURSE OF THESE EXPERIMENTS

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Translation of "Durch Direkte Mikroskopische Beobachtung Ausgeführte Untersuchungen über das Wachstum des Coli-Bazillus. -- Die Periodizität der Bakteriophagenwirkung, von den bei diesen Untersuchungen Gemachten Beobachtungen aus Untersucht," Centralblatt für Bakteriologie, etc., I. Abt., Originale, Vol. 107, No. 1-3, pp. 1-34, 1928

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION
WASHINGTON, D. C. 20546 MAY 1971

INVESTIGATIONS INTO THE GROWTH OF *BACILLUS COLI* BY DIRECT MICROSCOPIC OBSERVATION.[1] THE PERIODICITY OF BACTERIOPHAGE ACTIVITY STUDIED ON THE BASIS OF OBSERVATIONS MADE IN THE COURSE OF THESE EXPERIMENTS

K. A. Jensen[2]

ABSTRACT: After a brief survey and critique of the various methods of quantitative determination of bacterial growth, the most important works on conditions of bacterial growth are discussed. A technique is then described which is based on Oerskov's pure-culture method and by which it is possible to describe and measure bacterial growth by direct microscopic observation.

In order to facilitate an understanding of this article, let me first give /1* a brief account of the extant literature concerning normal bacterial growth and the methods of determining it.

The methods used to date may be classified into three main groups, each of which is based on a different fundamental method: Group I, in which only the number of germs capable of development is determined, is based on Koch's plate method and the Pasteur-Miquel dilution method. Group II, in which the total number of germs is determined, is based on the counting-chamber principle. This group includes Klein's field-of-vision method [1], Wright's counting method [2], the nephelometric and volumetric methods, and weighing of bacteria. Group III, in which growth and morphology are observed by direct microscopy (Barber [3], Barnewitz [4], and K.A. Jensen [5]).

The use of this last technique has the great advantages that 1) bacteria capable of development are distinguished from those incapable of growth, and 2) the rate of division and morphology of the bacteria can be observed throughout

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[1]This part of the paper is available in a more detailed form in my Habilitationsschrift [probationary thesis] *Untersuchungen über die Normalen Wachstumsverhältnisse des* Bacillus coli (Studies of the Normal Conditions of Growth of *Bacillus coli*].

[2]Of the State Serum Institute, Copenhagen, Director: Dr. Th. Madsen.

*Numbers in the margin indicate pagination in the foreign text.

the whole period of growth. It is thus also a great shortcoming of these methods of Group I that only the number of the germs capable of development is observed. That this is far from corresponding to the number of living germs, especially in young cultures, is clear from the works on the subject (Winterberg [6], Klein [7], Hehewert [8], Dichtl [9], Zelikow [10], Wilson [11], and Heckscher [12], among others). The methods of Group II have an equally great shortcoming, in that we get no information as to how many bacteria are living or dead. It is only by a combination of the methods, which, however, greatly increases the difficulty of the technique, that these shortcomings are to a certain extent overcome.

After this brief discussion of th various methods, I should like to go on to discuss our present knowledge of normal bacterial growth.

Let us merely mention that Naegeli and Schwendener [13] were the first who attempted to measure the rate of growth of bacteria, -- by determining the amount of acid developed during the growth of a culture, on the assumption that it is proportional to the content of active cells. Later works, first of all the basic investigations of Buchner, Longard, and Riedlin [14], Max Müller [15], and /2 Barber [3], and also the works of Winterberg [6], Hehewert [8], Rahn [16], Penfold [17], Lane Claypon [18], Heckscher [12], Wilson [11], Reichenbach [19], Sherman and Albus [20], and Schultz and Ritz [21] have yielded the following information concerning the normal growth:

When a substrate is inoculated with bacteria, some time first elapses before the rate of division reaches a maximum (latent time) (Von Buchner, Longard, and Riedlin [14], Max Müller [15], Barber [3], and Penfold [17]). This latent time varies with the age of the culture, increasing as the age increases. In this connection it must be particularly emphasized that a culture that is in the stage of logarithmic growth (see below) shows no latent time after reinoculation (Barber [3], Penfold [17]). Once the latent time is past, a period begins in which the rate of division is uniform and maximal, the *stage of logarithmic growth* (Max Müller [15], Barber [3], Lane-Claypon [18], Penfold [17]). After the cessation of this phase of growth the rate of growth declines until the culture has almost finished its development.

With regard to the viability of the cultures the authors are by no means in

agreement. While Barber [3] has demonstrated by an extraordinarily reliable method that young cultures exhibit a percentage of growth approaching 100%, there are numerous observations extant which show that in a comparison between the plate method and bacteria counting a far lower value is always obtained by the former, even in the case of young cultures; i.e., the number of germs in young cultures capable of development falls far below 100% (Winterberg [6], Klein [7], Hehewerth [8], Dichtl [9], Zelikow [10], Wilson [11], Heckscher [12].

Thus Hehewerth obtained a figure for capability of development for an eight-hour bouillon culture that was only 23.2%, while Winterberg found 42 to 70% for an 18-hour *B. pyocyaneus* culture, despite the fact that under direct microscopy all the bacteria were motile. These investigators attempted to explain this strange disparity between plate method and counts in the following way: 1) agglutination of the bacteria, so that in the plate method only one colony developed from several germs; 2) variation in the resistance of the bacteria. This assumption was supported to a great extent by the experiments of Reichenbach [19], Sherman and Albus [20], and Schultz and Ritz [21], which showed that young cultures are less resistant to heat and cold and to hypertonic and hypotonic salt solutions than older cultures.

Everybody agrees that the capability of the culture for development decreases steadily after it has reached a certain age. With regard to the morphology of the bacteria during the growth, it is a known fact that the size of bacteria in old cultures decreases; this is in agreement with Ficher's observation that the bacteria in the interior of a colony are smaller than those in the marginal zone. It has also been established by Heckscher in dry preparations stained by Klein's method that this change in size sets in quite suddenly and simultaneously with a decrease in rate of growth.

After this brief orientation, let me proceed to mention my own investigations.

The experimental technique used was based on Oerskov's ingenious pure-culture method [22]. Since the entire procedure involved has already been described in detail in my study of the effect of metallic salts on bacterial growth, I will only mention the principle itself here.

By Oerskov's method the bacteria can be observed through a powerful dry

lens and draw in the bacteria sown on the surface of an agar cube by means of a quadratic ocular micrometer. The cube of agar is placed in the moist chamber in the thermostat and the same field of vision is returned to after various periods of time. This makes it possible 1) to observe the morphology of the bacteria during the period of growth, 2) to obtain an expression for the rate of growth every half-hour or hour in counting the bacteria, and 3) to find out the percent of germination by determining the number of bacteria developing.

In the experiments reported below I have used the term "index for ½, 1, 1½ etc. hours" as a measure of the rate of growth; this represents the number of bacteria that have developed from one bacterium in the course of ½, 1, or 1½ hour.

Series 1. Determination of the Latent Time of *Bacillus Coli* and Its Initial Rate of Growth

In this series of experiments I observed and measured the rate of growth of/3 each individual bacterium until, after about three hours' growth, the colony had attained a size that prevented exact counting. For the sake of brevity, in this and later series of experiments only individual experiments are reported, but all the material is available to interested parties in the abovementioned Habilitationsschrift.

In the tables of this series of experiments the numbers above the line indicate the number of individual bacteria after various periods of growth and the numbers below the line the average growth of all the bacteria. In the curves the number of bacteria is plotted logarithmically along the ordinate, while the abscissa gives the time in hours. The solid line indicates the average growth, the broken line the growth of individual bacteria; especially those bacteria were plotted in here that showed the greatest deviations from the average growth.

Examination of the results of Experiment No. 1 will show that the bacteria inoculated immediately begin to divide, and that they grow from the very first with uniform and optimal speed. (The little break in the curve is due to the fact that the cube of agar was cold at the time of the inoculation.)

Experiment No. 2, on the other hand, shows that a certain time elapses before the onset of bacterial division; the bacteria do not begin to multiply until

they have survived a *latent period.* The table also shows that the *individual bacteria* exhibit different latent times, and that as soon as fission has begun, they appear to multiply at maximal speed. In Series 3 we find that the latent time of the old culture is still longer and that the irregularities in growth of the individual bacteria no longer show up. I have called the time during which the bacteria do not multiply at all as the *absolute latent time.* Morphological /4 observation has shown, however, that the bacteria do not remain unchanged during this period, for the small bacteria *(small permanent forms)* inoculated in Series 2 and 3 changed in the course of the absolute latent time into *large growth forms.* On the other hand, in Experiment 1 they consisted of large forms at the time of reinoculation, and fission set in immediately without a latent period. Now if we consider the average growth (Curves 2 and 3), we find an absolute latent time here, too, which must, of course, correspond to the shortest absolute latent time for all the bacteria under observation; but in Experiments 2 and 3 we shall also find a period in which the bacterial fission has begun, but has by no means become maximal. This period of time I have called the *relative latent time*, and it is due to the fact that the individual bacteria of the culture have an absolute latent time of varying length. After the end of the latent time the bacteria subdivide at a maximal and uniform rate such that the bacteria count measured at half-hour intervals shows a straight line when plotted in a logarithmic system (Curves 1, 2, and 3). Because of this mode of growth this period is called the *logarithmic growth stage.*

Series 2. Determination of the Further Course of Growth

With the experimental technique used thus far it was possible to observe bacterial growth only for the first two or three hours. It has been possible to overcome this difficulty, however, by a change in technique in which, while it /5 can hardly be said that the actual pattern of growth is observed, a curve nevertheless results that must run parallel to that pattern. As was shown in Series 1, a bacterium grows without latent time if it is derived from a culture in the stage of logarithmic growth, while when cultures not in that stage are inoculated a latent time must be gone through before the beginning of growth. It was also shown that this latent time increases with the age of the culture. If we measure the growth of the culture under examination at short intervals during the first

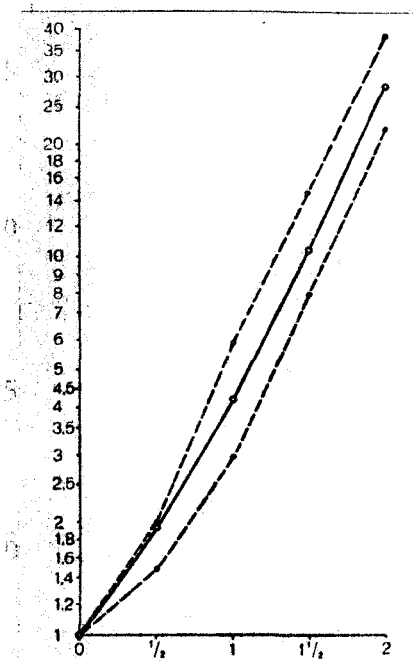
half-hour and first hour, and if we could determine the latent time in that way, the growth curve could be constructed from those data. This was possible by the technique described below.

TABLE I.

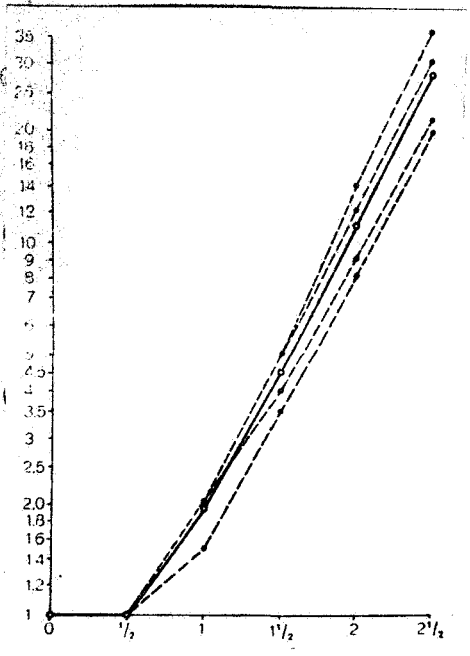
Experiment 1 3-Hour *Coli* Culture					Experiment 2 18-Hour *Coli* Culture						Experiment 3 $2^3/_4$-Day *Coli* Culture								
0	½	1	1½	2hr	0	½	1	1½	2	2½hr	0	½	1	1½	2	2½	3	3½	4hr
1	2	6	15	38	1	1	2	5	12	30	1	1	1	1	2	4	10	23	
2	4	8	16	38	1	1	2	5	13	30	1	1	1	2	4	9	20	46	
1	2	5	13	32	1	1	2	5	13	33	1	1	1	1	2	4	8	20	
2	4	8	18	54	1	1	2	5	14	32	1	1	1	1	1	1	2	3	4
2	4	8	18	55	1	1	2	4	9	21	1	1	1	1	1	1	2	2	2
2	4	9	30	71	1	1	2	5	11	30	1	1	1	1	2	3	5	11	24
2	4	8	21	55	1	1	2	4	11	29	1	1	1	2	4	7	14	30	
2	4	9	23	62	1	1	2	5	14	36	1	1	1	2	4	8	17	33	
2	3	6	16	44	2	2	3	7	16	40	1	1	1	1	1	1	1	2	
2	4	8	20	58	1	1	2	5	11	28	1	1	1	1	2	4	8	16	
2	4	9	25	63	1	1	2	4	12	32	1	1	1	1	1	1	1	2	4
2	4	10	25	66	2	2	4	9	20	47	1	1	1	1	1	2	3	6	13
2	4	8	17	52	2	2	4	10	26	60	1	1	1	1	1	1	2	4	8
2	4	8	17	55	2	2	4	9	23	52	1	1	1	2	4	8	15	31	
					2	2	4	9	21	50									
1.00	1.96	4.23	10.54	28.34	1.00	1.00	1.95	4.55	11.3	27.5	1.00	1.00	1.00	1.29	2.14	3.86	7.71	16.4	

Some agar dishes with the same amount of substrate were inoculated as uniformly as possible and so densely that after six hours of growth the colonies came together. From one agar dish a cube was cut immediately after inoculation and growth on that cube was observed by the method used earlier for as long as possible. The other dishes were put in the constant-temperature bath and 1½ hours later one of them taken out and a part of the surface rinsed off with 3 drops of distilled water, a new cube of agar inoculated with the rinse water, and the rate of growth determined after ½-1 hour. After 2, 2½, 3, and 3½ hours and up to the termination of the experiment, subcultures were started in this way from the original dishes and their energy of growth determined every half

hour. Table II and Curves 4 and 5 show the result of the experiments.



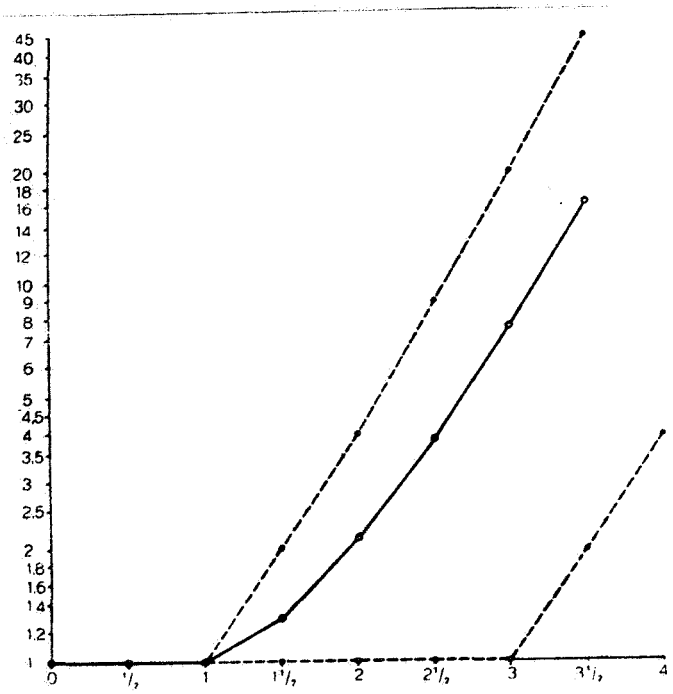
Curve 1. Experiment 1.



Curve 2. Experiment 2.

In these experiments the average growth of at least 30 bacteria was determined, while the bacteria that did not grow were ignored. (See the series of experiments on germinating capacity.) /6 The growth rate is given as a half-hour index. At the same time, the hourly index is given in the tables as a check on the small numbers obtained after half an hour's growth. The curves were drawn on the following assumption: by my mode of computation, index 1 denotes that no growth has taken place. The increase of the index above 1 is then the expression of the rate of growth, and this quantity is plotted on the ordinate, while the time in hours is given on the abscissa. In the tables the numbers above the line indicate the growth observed as in the series of experiments described earlier, but the numbers below the line indicate the index value after another inoculation from the original dishes in half-hour intervals.

By studying the table and the corresponding /7 curves it is possible, as in Series 1, to find an absolute and a relative latent time. When these are past, a period of growth sets in during which both the half-hourly and the hourly index are constant -- the definition of the logarithmic stage of growth. When this is over, there comes a period of rapidly declining rate of growth. The two series of experiments thus confirmed earlier observations concerning bacterial growth, since they show three well-marked phases of growth: 1) latent time (absolute and relative; 2) the logarithmic stage of growth; 3) the growth period with decreasing rate of growth.



Curve 3. Experiment 3.

Series 3. Studies of *B. Coli's* Capacity for Growth

As was mentioned in the introduction, in determining the content of bacteria capable and incapable of growth in a culture we have only a rather inadequate method available (simultaneous bacteria counts and plate method).

For that reason our knowledge of the capacity of a culture for growth is very incomplete. The only sure observations in this field are those made by Barber /8 [3] in direct microscopic examination, by which it was shown that quite young bacteria were all capable of germination after transfer to a new substrate. It seems to me, however, that the experiments described below have brought us closer not only to an understanding of this problem, but also to the whole problem of bacterial growth. The technique was the same as in Series 2, in which the rate of growth of the cultures was observed from just after inoculation to an age of 24 hours. The bacteria plotted were observed until no further growth could be expected.

The results of these experiments did not seem very probable to me at first, because the three curves showed two germination maxima, but this behavior was repeated in numerous experiments. It was only later that I discovered the explanation of this problem through a series of experiments concerning the morphology of the bacteria during their growth.

If we consider the quite young cultures corresponding to the first germination optimum (e.g., the two to three-hour-old cultures in Experiment 6), we find that the bacteria immediately after smearing on the cubes of agar are large and strongly light-refracting; these forms, as mentioned earlier, form the large growth forms, all of which grow. In the next subsequent period (between the third and sixth hour in Experiment 6), among the large growth forms there are

a larger or smaller number of big, not very light-refractive bacilli, which appear as pale, almost transparent shadows (*big bacteria shadows*). In observing the fate of these shadows we found that under these growth conditions they were not capable of germination; this had already manifested itself in the preceding studies in a decreasing capacity for germination of the culture. At the end of the stage of logarithmic growth the big bacteria quite suddenly changed into little, strongly refractive forms *(small growth forms)*, all of which were capable of germination, and at the same time the bacteria shadows disappeared. This was followed by an increase in the percentage of germination up to nearly 100, corresponding to the second optimum. Finally a new phase of growth set in, during which the capacity for germination decreased with the age of the culture.

TABLE II. (EXPERIMENTS 4 AND 5)

GROWTH OF A FRESHLY ISOLATED *COLI* CULTURE 18 HOURS OLD. THE NUMBERS ABOVE THE LINE INDICATE THE OBSERVED GROWTH, AS DESCRIBED ABOVE. THE NUMBERS BELOW THE LINE INDICATE THE INDEX VALUES AFTER REPEATED INOCULATION FROM THE ORIGINAL PLATES AT HALF-HOUR INTERVALS.

EXPERIMENT 4

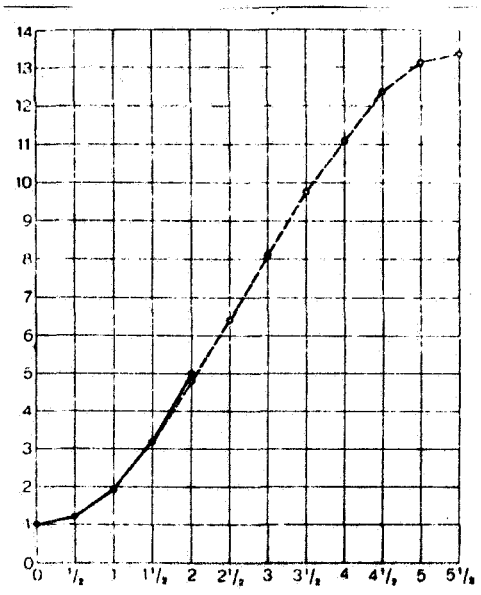
Growth after	0	½	1	1½	2	2½	3	3½	4	4½	5 hr.
No. of bacteria	30	36	61	141	396 ?						
Index	1	1,2	1,69	2,31	2,8						
Half-hour index				2,56	2,65	2,68	2,64	2,35	2,28	1,77	1,20
Hourly index				7,0	6,4	6,2	7,2	5,8		5,2	2,31

EXPERIMENT 5

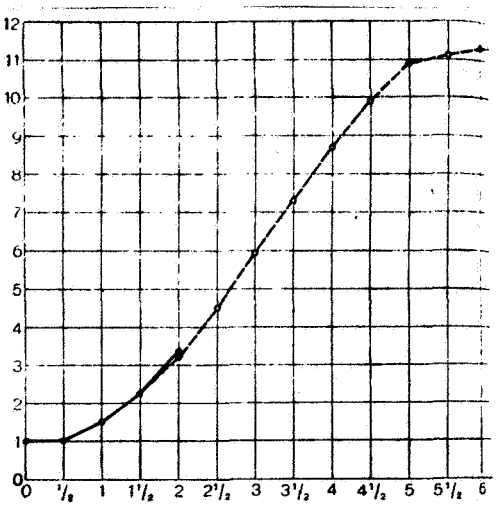
Growth after	0	½	1	1½	2	2½	3	3½	4	4½	5	6	7	8	15	24 hr.
No. of bacteria	26	26	39	82	197											
Index	1	1	1,5	2,1	2,1											
1/2 hour index				2,1	2,44	2,26	2,17	2,16	1,88	1,74	1,34	1	1	1	1	1
1-hour index				4,32	5,9	4,64	4,50	4,65	4,35	3,92	2,3	1,45	1,43	1,22	1	1
1 1/2-hour index												2,89	2,94	2,05	1,22	1,30
2-hour index															3,98	4,01

Commas indicate decimal points

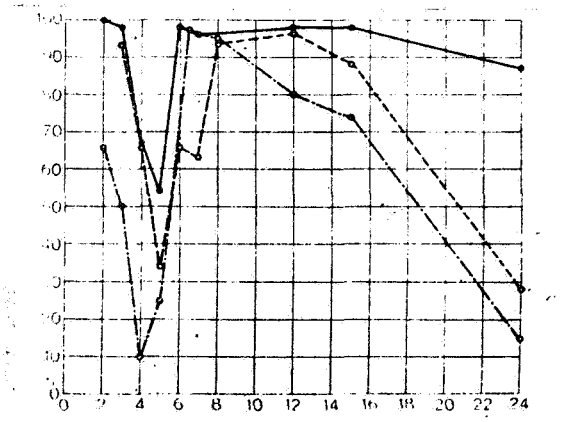
Numbers on the ordinate, as mentioned in the text, indicate the growth. Numbers on the abscissa indicate the time in hours. The solid line of the curve corresponds to the numbers above the line, the broken line to those below it.



Curve 4. Experiment 4.



Curve 5. Experiment 5. Abscissa, Ordinate, and Curve Indicate the Same Conditions as in Curve 4.



Curve 6. Experiments 6, 7, and 8. The Numbers Along the Ordinate Indicate the Percentage of Germination; Those of the Abscissa Indicate the Time in Hours.

——— Experiment 6
-·-·-·- Experiment 7
------- Experiment 8

In the three series of experiments described above, I have discussed for each series independent of the others the various conditions that prevail during the growth of *Bacillus coli* on agar. In order to give an objective picture of the growth of the bacteria, the mutual relationships of these conditions had to be investigated in greater detail, and for that reason I investigated the rate of growth, the germinating capacity, and the morphology of the bacteria simultaneously in the series of experiments described below. /9

TABLE III

Experiment No. 6 Starting Culture 18 Hours Old. Freshly Isolated Coli Strain		Experiment No. 7 Starting Culture 18 Hours Old. Old Laboratory Strain (Coli B)		Experiment No. 8 Starting Culture 1 Week Old. Old Laboratory Strain (Coli B)	
Age of the Culture	Percentage of Germination	Age of the Culture	Percentage of Germination	Age of the Culture	Percentage of Germination
2 hours	100	2 hours	66	2 hours	Latent time not past
3	98	3	50	3	93
4	67	4	<10	4	66
5	54	5	25	5	34
6	98	6½	97	6	66
7	96	8	95	7	63
12	98	12	80	8	94
15	98	15	74	12	96
24	87	24	15	15	88
				24	28

Series 4. Investigations of Rate of Growth, Germinating Capacity, and Morphology of *Bacillus Coli* Conducted Simultaneously

The experimental technique was the same as in Series 2; i.e., inoculation of the original dishes and secondary inoculation of the cubes of agar. During the experimental period the rate of growth of the bacteria capable of germinating, the percentage of germination, and the morphological picture were determined at half-hour intervals.

Tables IV and V and Curves 7 and 8 show the results of the experiments. The numbers above the line in the tables correspond to the numbers above the line in Series 2 (see Table II), and were determined by observing the growth on a cube of agar cut from the original dish. The curves were plotted in the same /10 way as in Series 2. On the ordinate, as in Curves 4 and 5 above, the percentage of germination is shown on the first scale and the index values on the second, while the time is given along the abscissa. The solid line represents the growth curve, the broken line the percentage of germination. The figure above the curve indicates the growth as a pure approximation, so that the vertical lines of this /11

graph (Curve 7) give the relative size at every point of time. The partly wavy and partly straight line at the top indicates the culture's bacteria shadow content, the waves indicating the period of time during which those forms are found and the straight line the period of time during which no shadows are present (see Tables IV and V).

TABLE IV (EXPERIMENT 9). SURFACE OF THE AGAR INOCULATED WITH A FIFTEEN-HOUR-OLD FRESHLY ISOLATED COLI CULTURE

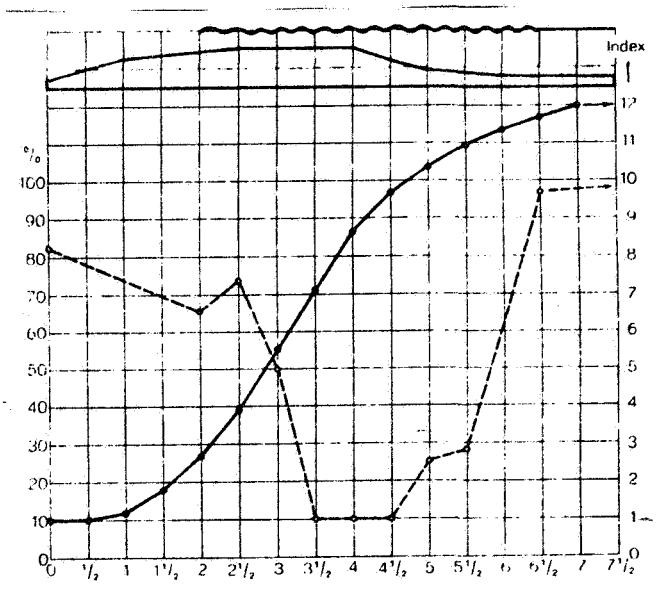
Age in Hours	Morphology of the Bacteria Observed Immediately After Inoculation	Index			Percentage of Germination		
		½ Hr.	1	1½	½ Hr.	1	1½
0 (Original culture observed on a dish)	Small growth forms					100	
½	Bacteria increased in size, some dividing	1.00					
1	Large growth forms	1.95					
1½	" " "	2.33					
1½	" " "	2.50	5.9		100	100	
2	" " "	2.68	6.2		95	95	95
2½	" " "	2.51	6.8		100		
3	Large growth forms, a few large bacteria shadows	2.24	5.9		96	96	96
3½	Large growth forms, numerous bacteria shadows	2.29			57	57	57
4	As above	1.73	4.0		60	60	60
4½	Bacteria decreasing in size, numerous large shadows	1.40	2.2		44	44	44
5	Mostly small growth forms, a few shadows	1.50	2.7		73	86	86
6	Small growth forms, no shadows	1.16	2.0	5.0	16	80	96
8	As above	1.00	1.34	2.7	0	30	97

In this way I got all the growth conditions into a single system of curves which brings out clearly the relationship in time. For the growth curves, the germination curves, and the morphology the experiments produced the same results in general as Series 1, 2, and 3. If we consider the relationship between these features, the curves show that immediately after inoculation the bacteria are

TABLE V (EXPERIMENT 10). SURFACE OF THE AGAR INOCULATED WITH A FIFTEEN-HOUR-OLD CULTURE OF AN OLD LABORATORY STRAIN

Age in Hours	Morphology of the Bacteria Observed Immediately After Inoculation	Index			Percentage of Germination			
		½ Hr.	1	1½	½ Hr.	1	1½	2
0 (The original culture observed on a dish)	Small growth forms, a few bacteria shadows							
½	Bacteria increasing in size, no division	1.00						
1	Large growth forms; fission beginning	1.23						
1½	As above	1.52						
2	As above	1.90						
2	Large growth forms, a few large shadows	2.30	5.9		66	66		
2½	As above	2.53	6.8		74	74		
3	As above, but more bacteria shadows	2.58	6.5		50	50		
3½	Nearly all large shadows; otherwise large growth forms	2.58	6.4		<10	<10		
4	Nearly all large and small shadows; large and some small growth forms	2.00			<10	<10		
4½	As above, but no large growth forms and only small ones	1.73	4.5		<10	<10		
5	Bacteria decreasing in size	1.58	3.6		25	25		
5½	Mainly small shadows; otherwise only small growth forms	1.41	3.4		12	28	28	
6½	Small growth forms, no shadows	1.35	3.2		36	97	97	
8	As above	1.00	2.19		0	95	95	
12	Small growth forms, a few small shadows	1.00	1.09	2.87	0	8.6	80	80
15	As above	1.00		2.42	0	10.6	74	74
24	Small permanent forms, numerous small shadows	1.00		2.23	±	0	15	15

small (permanent forms) and that they grow in the course of the latent time into large growth forms, with maximal rate of fission, which remains constant for a certain period (the stage of logarithmic growth). At a certain point in time



Curve 7. Experiment 10.

during this period the large bacteria shadows begin to appear, so that the germinating capacity declines. A fairly rapid increase in these bacteria shadows incapable of germination follows, until quite suddenly a transformation of the large, fast-growing growth forms into small forms with a lower rate of fission sets in. This makes a break in the growth curve in the form of a bend in the horizontal direction. Simultaneously with this transformation the bacteria shadows disappear, so that the growth curve rises to the second optimum. The experiments described below show clearly that growth in broth takes place in exactly the same way.

Series 5. Investigations of the Bacteria Shadows

In the experiments mentioned above, I described the microscopic observation of the bacterial growth after inoculation on the surface of the agar without detailed criticism. It is conceivable, however, that the shadows mentioned and the simultaneous decrease in percentage of germination might be a consequence /14 of purely mechanical influences during the preparation of the suspension and in the inoculation process, and not a biological phenomenon during the growth. Even if this is not the case, it is interesting that in all the experiments the shadows make their appearance in a certain period of growth, a fact that would have to be interpreted in any case as showing a lower resistance of the bacteria in that period. This also appeared to be in complete agreement with the experiments of Schultz and Ritz [21] and of Schermann and Albus [20], which similarly showed a period in which in young cultures the resistance of the bacteria to harmful influences was decidedly lowered. Thus from whatever point of view we regard this phenomenon, it seems to me it is evident that there must be differences in the original culture corresponding to the forms observed on the cubes

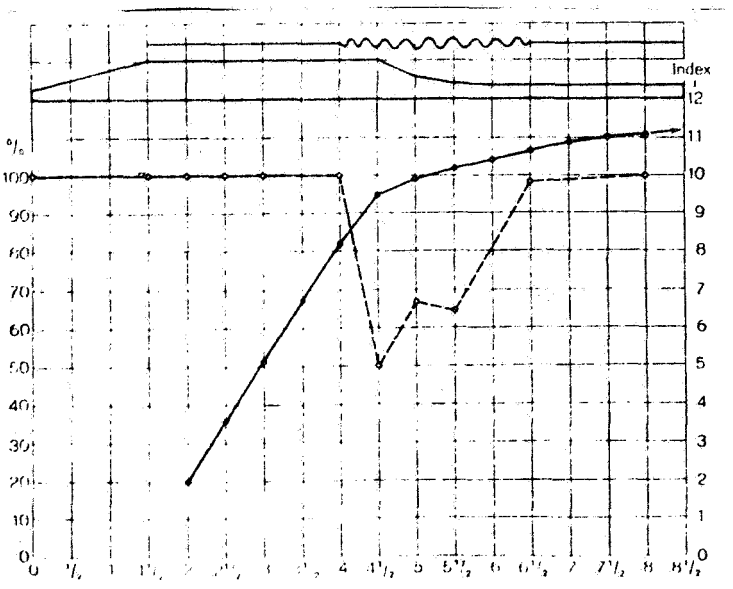
of agar. It was found by experiment that the suspension medium played no part in the appearance of the shadows, since gently strewing the material dry on the original dish produced the same result. I therefore proceeded to investigate whether bacteria shadows were present in the primary culture without inoculation, and in so doing arrived at the core of the problem.

TABLE VI (EXPERIMENT 11). BROTH INOCULATED WITH A FIFTEEN-HOUR-OLD CULTURE OF A FRESHLY ISOLATED COLI STRAIN

Age in Hours	Appearance of the Broth	Morphology of the Bacteria Immediately After Inoculation	Index			Percentage of Germination		
			½ Hr.	1	1½	½ Hr.	1	1½
1½	Clear	Large growth forms	1.94	4.8		100	100	
2	"	" " "	2.65	6.8		100	100	
2½	"	" " "	2.56	6.6		100	100	
3	"	" " "	2.53	6.3		100	100	
4	Trace of growth	" " "	2.32	5.6		100	100	
4½	Slight cloudiness	Mainly large, a few small growth forms, numerous shadows	1.83	3.8		50	50	
5	More cloudiness	Mainly small growth forms, fewer shadows	1.24	2.02		67	67	
5½	Great cloudiness + air	As above	1.32	2.01		36	65	
6½	As above	Bacteria continuously in size; no shadows	1.20	1.60	1.95	25	94	98
8	As above	Bacteria very small, strong-light-refractive	1.05	1.55	1.95	13	87	100

The experiment was carried out by staining the bacteria on the surface of the agar with one-to-one diluted carbol-fuchsin *in situ* to be observed subsequently with the immersion lens. At the same time, the morphology of the cultures after inoculation on agar was studied in the usual way.

It was found that the bacteria of young cultures with large growth forms but without shadows stained weakly and uniformly. At the point of time when the bacteria shadows began to appear, however, their stainability decreased so that when the shadows were most numerous the bacteria stained weakly everywhere. In

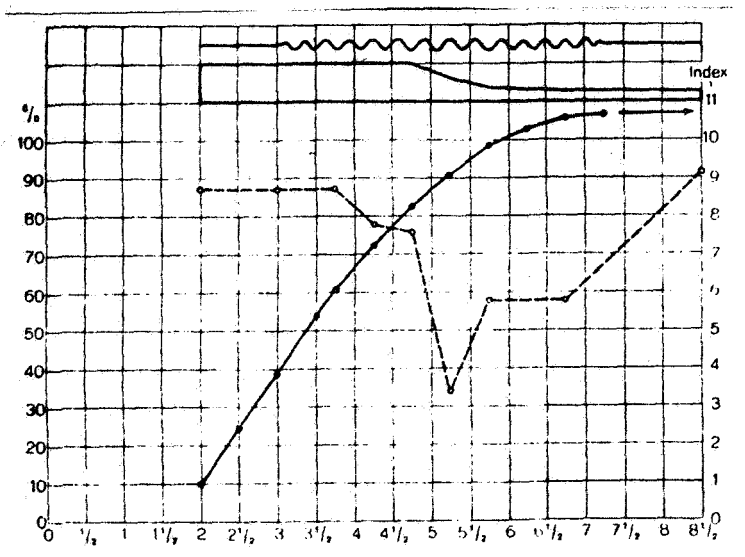


Curve 8. Experiment 11.

the cultures, among the uniformly stained bacteria, quite pale and completely unstained forms were found simultaneously, the number of which was roughly equal to the number of bacteria shadows. Toward the end of the phase with the bacteria shadows, some bacteria again began to take up the stain more strongly, so that the difference stood out very distinctly, and gradually all the bacteria could be stained fairly uniformly and strongly. This phase corresponded to the second germination optimum with the small bacteria without shadows.

TABLE VII (EXPERIMENT 12). BROTH INOCULATED WITH FIFTEEN-HOUR-OLD CULTURE OF AN OLD LABORATORY STRAIN (COLI B)

Age in hours	Appearance of the broth	Morphology of the bacteria immediately after inoculation	Index		% of germination	
			½ hr.	1 hr.	½ hr.	1 hr.
2	Clear	Large growth forms, a single large shadow	2.46	5.8	87	87
3	"	As above	2.44	6.7	87	87
3 3/4	Incipient	Large growth forms, scattered large shadows	2.18	5.3	86	86
4¼	Moderately cloudy	Mainly large growth forms and scattered large shadows; also scattered small shadows	2.04	4.5	78	78
4 3/4	Much clouded	Large and small growth forms and some large shadows; scattered small ones	1.79	---	76	76
5 ¼	Much clouded + air	Mainly small growth forms, a few large and numerous small shadows	1.78	3.6	32	34
5 3/4	As above	The bacteria continously decrease in size. No large shadows, the number of small ones decreasing	1.48	3.3	30	58
6 3/4	As above	As above	1.10	2.44	9.2	58
8 ½	As above	The bacteria are now quite small, short, and strongly light-reactive. No shadows	1.00	2.30	0	91



Curve 9. Experiment 12.

However this is to be explained, it was found that in the period of rapid growth the bacteria are weakly stainable, and that in that period an actual inequality of capacity for taking up stain is present in the different bacteria of the same colony, such that the most weakly staining bacteria correspond to those which manifest themselves as shadows in ordinary examination. In my opinion this can only be due to a biological difference between the bacteria, and by this finding I believe I have shown that the bacteria shadows are a biological phenomenon connected with growth.

It then remained to investigate the factors by which it is brought about that the morphology of the cultures changes in this way during the growth, and in particular what effects the transformation of large growth forms into small ones and the occurrence of the shadows.

This transformation of the culture, in my opinion, can be caused by two factors: 1) The formation of metabolic products which may have a harmful effect on the fast-growing large growth forms, and thus bring about the formation of the less viable bacteria shadows, but which cannot effect that result later, when the bacteria have changed into the small, more resistant growth forms; and 2) *exhaustion of the nutrient medium with reference to some material readily ac-*/15 *cessible for building up the bacterial cell.*

The second assumption is supported by various observations made in the course of my earlier observations, namely: 1) that the shadows that develop at the time when the bacteria are changing from large to small growth forms are apparently forms poor in protoplasm, which seem not to have had sufficient material

to build up their structure; 2) that acid formation sets in in the broth culture toward the end of the stage of logarithmic growth; and 3) that when *Bacillus coli* is inoculated on water agar containing traces of broth, the growth proceeds very slowly, the period with the large growth forms being either entirely lacking or of very brief duration. It is also of special interest that in this case the bacteria appear throughout their period of growth as only slightly refractive bacilli, reminiscent of the shadows.

In order to investigate which of the abovementioned two factors caused the transformation of the culture, comparative experiments were set up on the growth in fresh broth and in a broth culture twenty-four hours hole that had been filtered and neutralized to pH 7.1.

TABLE VIII (EXPERIMENT 13). FRESH BROTH (CONTROL) AND FILTERED 24-HOUR BROTH CULTURE (FILTRATE) INOCULATED WITH THE SAME AMOUNTS OF A 48-HOUR AGAR CULTURE *(BACILLUS COLI)*

Control		Filtrate	
Appearance of the Broth and Morphology of the Bacteria	Index ¾ Hr.	Appearance of the Broth and Morphology of the Bacteria	Index ¾ Hr.
1st Hour: Broth clear, small bacteria (latent time not past).	1.50	1st Hour: Broth clear, small bacteria	1.00
2nd Hour: Broth slightly opalescent, large growth forms, no shadows	3.10	2nd Hour: Broth clear, small bacteria, numerous shadows	1.23
3rd Hour: Medium cloudiness of the broth. Large growth forms, occasional shadows	2.97	3rd Hour: Broth clear. Mainly permanent forms. A few shadows	1.12
4th Hour: Broth much clouded and containing air. Bacteria reduced in size. A majority of small growth forms, a few shadows	1.91	4th Hour: Broth clear. No permanent forms	1.00

In Experiment 13 the two nutrient media were inoculated with equal amounts of a 48-hour agar culture, in Experiment 14 with the centrifugate of a two-hour broth culture. What was to be investigated here was how the large growth forms behaved in the two nutrient media and whether the old culture would run through the growth-phase forms discussed earlier in the two cases.

TABLE VIII [continued] (EXPERIMENT 14). FRESH BROTH (CONTROL) AND FILTERED 24-HOUR CULTURE (FILTRATE) INOCULATED WITH THE SAME AMOUNTS OF A 48-HOUR AGAR CULTURE *(BACILLUS COLI)*

Control		Filtrate	
Appearance of the Broth and Morphology of the Bacteria	Index ¾ Hr.	Appearance of the Broth and Morphology of the Bacteria	Index ¾ Hr.
1st Hour: Broth clear, large growth forms, no shadows	3.14	1st Hour: Broth clear, large growth forms, several shadows	1.80
2nd Hour: Medium clouding of the broth, large growth forms, few shadows	2.91	2nd Hour: Broth clear. Small growth forms and permanent forms; numerous shadows	1.31
3rd Hour: Broth much clouded and containing air; large growth forms, numerous small growth forms, some shadows	1.63	3rd Hour: Broth clear, small permanent forms, some shadows	1.16

In these experiments the first thing one notices is the great difference in growth between fresh and old filtered broth. Thus it is evident from Experiment 13 that the bacteria inoculated into fresh broth follow the abovedescribed phases precisely (though because of the abundant inoculation material, the logarithmic growth stage is very brief), while the culture in the filtered broth did not show these phases. Thus no large growth forms appear -- the decisive factor for the low index -- and the bacteria shadows, the small growth forms, and the permanent forms occur in rapid succession. Experiment 14 shows the same behavior, though /16 in the filtered broth the large growth forms do not disappear in the course of 1 hour; but from the index it is clear that even at this point in time the bacteria show a reduced growth energy, and accordingly the shadows have begun to appear. The conditions observed may, of course, be due either to an inhibition of growth in the filtered broth because of insufficient nourishment or to an injury to the bacteria by metabolic products.

For that reason the experiment was repeated with the sole change that 10% of fresh broth was added to the filtered broth, on the assumption that the inhibition of growth would occur in this case, too, if it had been brought about by the metabolic products. The results of these experiments are shown in Table IX.

TABLE IX (EXPERIMENT 14). FRESH BROTH (CONTROL) AND A FILTERED 24-HOUR-OLD BROTH CULTURE WITH THE ADDITION OF 10% FRESH BROTH (FILTRATE) INOCULATED WITH THE SAME AMOUNT OF A 24-HOUR-OLD AGAR COLI CULTURE

Control		Filtrate	
Appearance of the Broth and Morphology of the Bacteria	Index ¾ Hr.	Appearance of the Broth and Morphology of the Bacteria	Index ¾ Hr.
1st Hour: Broth clear, large growth forms, no shadows	3.14	1st Hour: Broth clear, large growth forms, some shadows	1.80
2nd Hour: Medium cloudiness of the broth; large growth forms, a few shadows	2.91	2nd Hour: Broth clear; small growth forms and permanent forms and numerous shadows	1.31
3rd Hour: Broth very cloudy + air. Large growth forms. Numerous small growth forms. Some shadows.	1.63	3rd Hour: Broth clear, no permanent forms, a few shadows	1.16

EXPERIMENT 15. FRESH BROTH (CONTROL) AND A FILTERED 24-HOUR-OLD BROTH CULTURE WITH THE ADDITION OF 10% FRESH BROTH (FILTRATE), INOCULATED WITH THE SAME AMOUNT OF A 2-HOUR-OLD AGAR COLI CULTURE

Control		Filtrate	
Appearance of the Broth and Morphology of the Culture	Index ¾ Hr.	Appearance of the Broth and Morphology of the Culture	Index ¾ Hr.
1st Hour: Broth clear, large growth forms, no shadows	2.76	1st Hour: Broth clear, large growth forms, no shadows	1.85
2nd Hour: Medium cloudiness of the broth, large growth forms, few shadows	2.50	2nd Hour: Broth slightly clouded. Small growth forms, but also a few large growth forms, numerous shadows	1.76
3rd Hour: Great cloudiness + air; the bacteria decrease in size, but numerous large growth forms are still present. Numerous shadows	1.98	3rd Hour: Medium cloudiness. No growth forms and occasional permanent forms. Rather few shadows	1.37

This last experiment showed that the growth in filtered broth with an addi-/17 tion of 10% fresh broth proceeds almost as well during the first two hours as in the control brorth, and with the same phases (this is indicated by the index, by the cloudiness, and by the morphological picture). For that reason I am of the opinion that these experiments can be interpreted only on the assumption that the lack of some nutrient substance causes the transformation from large to

small bacteria with the shadows as an intermediate stage, since it can hardly be assumed that the slight dilution of the metabolic products can inhibit their harmful effect on the bacteria.

Some experiments in growing *Bacillus coli* on low-nutrition media showed the same pattern. The experimental procedure for that purpose was as follows:

A thin layer of water agar with the addition of 5% broth was poured and inoculated with a three-hour-old broth culture. The first two hours the bacteria grew as large, strongly refractive growth forms, but after 2½ to 3 hours both the size and the light refraction decreased. Two cubes were then cut from the cultures and one placed on ordinary agar and the other on water agar. For the first half hour the morphology of the two cultures was alike, but thereafter a definite difference was detected; the bacteria on water-agar cubes increased considerably in size and light refraction, while the reverse was true for the bacteria on water-agar-water-agar cubes. Two hours later this difference was still more pronounced, and this came out still more plainly when inoculations were done from the colonies. While the bacteria on water-agar-agar dishes had thus developed as large growth forms without shadows, the bacteria on water-agar-water-agar cubes showed up as sometimes large and sometimes small bacteria shadows.

A brief summary of these experiments will thus show:

1) The bacteria shadows and the transformation from large into small bacteria are produced by a deficient supply of nutrients;

2) The bacteria shadows are probably all capable of germination if they are not damaged in reinoculation; this is evident from the fact that all the shadows grew on the water-agar dish when an agar dish was placed beneath it.

3) The nutrient substance which transforms bacteria shadows into large growth forms, which may be regarded as identical with the substance mentioned earlier as necessary for the initial growth, is to be regarded as an agent diffusible through water agar. This capacity, in combination with the direct microscopic observation of bacterial growth, will surely be applicable to the systematic investigation of suitable nutrient media for the growth of bacteria. /18

Series 6. Comparative Quantitative Studies of Bacterial Growth in the Dish Procedure and in Direct Microscopy

I called attention in the introduction to the great difference found in determining the bacteria count of a culture by counting and by the dish method. Judging by my abovementioned experiments, I considered it evident that this difference was due to the lowered resistance of the bacteria with reference to reinoculation in the growth phase with the bacteria shadows. In order to study this in more detail, the following experiment was carried out:

A flask containing 100 cm^3 of broth heated to 37°C was inoculated with a measured amount of the coli culture and plates were prepared at the beginning of the experiment and each half-hour thereafter. The original culture was inoculated on a cube of agar in order to determine the rate of growth of the first hour, before the growth in the broth had become strong enough that secondary cultures could be made from it. That was possible after two to two and a half hours and was then done at hourly intervals. The plates were prepared with the greatest care. In order to avoid flowing together of the colonies on the under side of the plate, a thin layer of agar was first poured into the Petri dishes, which was then dried and put into the thermostat before use. The subdivision agar (1¾% agar) was kept in constant-temperature cabinets at 45°C between subdivisions. The dilution of the culture was done after shaking the broth flask hard with 0.9% salt water. The reinoculation was done as follows: First a layer of the subdivision agar was poured into the Petri dishes. The dishes were continuously swung back and forth, and when the agar began to take on a lumpy consistency, the culture was added. This procedure made it possible to add the culture to the subdivision agar while it was at a temperature of 38°C (measured with a thermometer). After the plates dried they were put into the constant-temperature cabinet for 24 hours and then counted. The dilutions of the culture were calculated so that the plates contained from two hundred to six hundred colonies each as a rule. The whole plate was then counted. Tables X and XI show the results of the experiments. The result of direct micrscopic observation is computed and entered in the tables in the same way as in the earlier experiments. The indexes for the plates were determined every hour. Curves 11 and 12 are the same as in Experiment 9; the index quantity is plotted along the abscissa, while the ordinate indicates the time. The broken-line curve indicates

the result of the plate procedure, the solid line that of the microscopic observation. The bacteria shadows and the size of the bacteria are indicated in the same way as in Experiment 9.

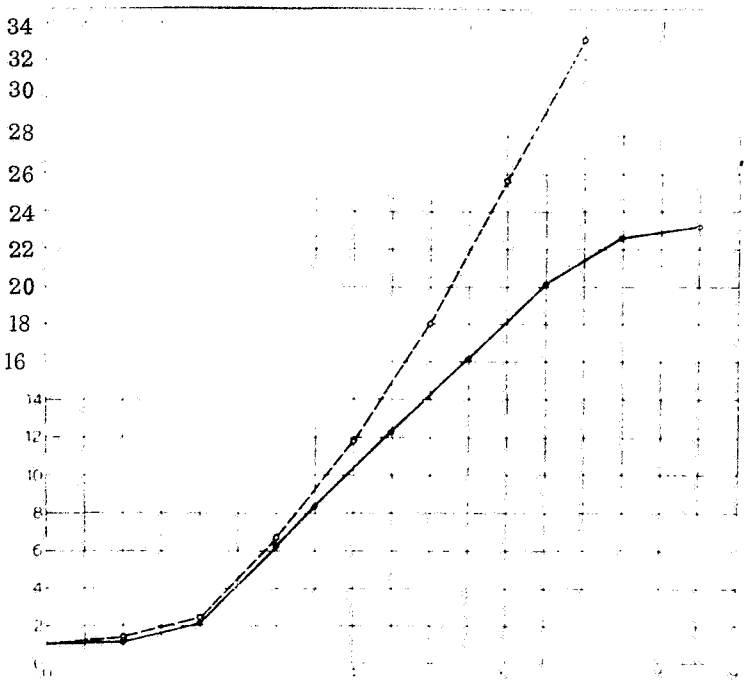
TABLE X (EXPERIMENT 17). GROWTH OF *BACILLUS COLI* (EIGHTEEN-HOUR-OLD AGAR CULTURE) IN BROTH, DETERMINED BY DIRECT MICROSCOPIC OBSERVATION AND BY THE PLATE PROCEDURE

Direct Microscopic Observation				Plate Procedure	
Hours	Morphology	Index ½ Hr.	Index 1 Hr.	Index 1 Hr.	Bacteria Count per cm^3
0					2,670
1			1.11	1.31	3,510
2			3.15	3.05	10,700
2½	Large growth forms, no shadows	2.22	5.1		
3			5.0	5.4	58,200
3½	Large growth forms, a few shadows	2.32	5.0		
4				6.1	356,800
4½	Large growth forms, a few shadows	2.20	4.8		
5				7.2	2,559,000
5½	Large growth forms, a number of shadows	2.11	5.0		
6				8.6	22,070,000
6½	Bacteria decreasing in size, fewer shadows	1.53	3.5		
7				8.2	182,000,000
7½	Small bacteria, fewer shadows	1.26	1.61		

From these experiments we see first of all (Curves 10 and 11) that the two /20
growth curves do not exhibit the same shape and slope. In both experiments the curves at first run parallel (in Experiment 17 for the first three hours, in experiment 18 for the first two hours). As may be seen from Tables X and XI, this occurs during the period when the growth was observed on the primary cube. When the rate of growth is determined by preparing secondary cultures and observing them by direct microscopy, we find that the index values come to be far lower than in the plate method. *Curves obtained by direct microscopy cannot be re-* /21
garded as a measure of the absolute number of bacteria.

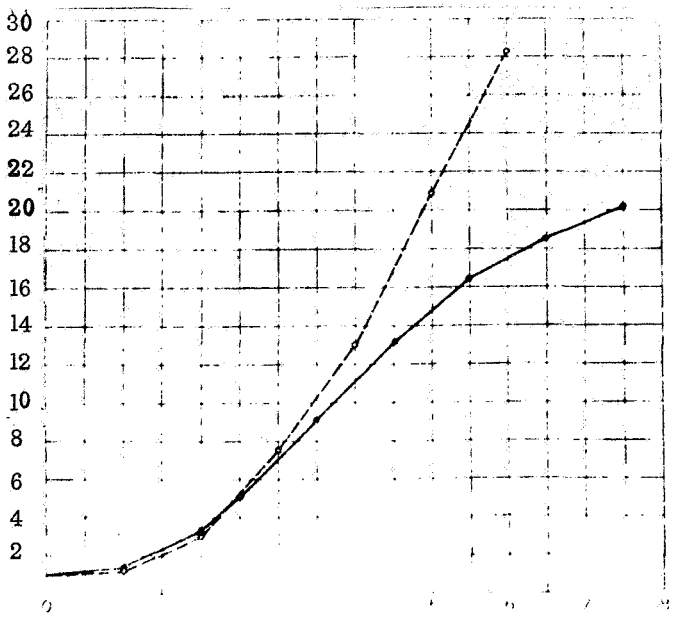
TABLE XI (EXPERIMENT 18). GROWTH OF *BACILLUS COLI* (SIXTEEN-HOUR-OLD AGAR CULTURE) IN BROTH, DETERMINED BY DIRECT MICROSCOPIC OBSERVATION AND BY THE PLATE PROCEDURE

Direct Microscopic Observation				Plate Procedure	
Hours	Morphology	Index ½ Hr.	Index 1 Hr.	Index 1 Hr.	Bacteria Count per cm^3
0					33,220
1			1.38	1.24	41,228
2			3.95	3.92	161,600
2½	Large growth forms, a few shadows	2.18	4.99		
3				5.4	878,000
3½	Large growth forms, some shadows	2.20	5.24		
4				6.4	5,650,000
4½	Bacteria decreasing in size, some shadows	1.99	4.11		
5				8.9	50,200,000
5½	Small growth forms, few shadows	1.75	3.15		
6				8.4	423,000,000



Curve 10. Experiment 17.

Upon comparing the duration of the various phases as determined by the two methods, we find from Experiment 17 that the latent time as determined by direct microscopy amounts to only two hours, but that determined by the plate method is four hours. In Experiment 18 the duration of the same period is again two and four hours respectively. If we compare the length and the times occupied by the



Curve 11. Experiment 18.

stage of logarithmic growth, we find that in direct microscopy in Experiment 17 it falls between two and 6½ hours after the beginning of the experiment, while in the plate method it is between 4 and ≧7 hours. In Experiment 18 the corresponding figures are 2 to 4½ and 4 to ≧6 hours respectively. This indicates that in the plate method the latent time is considerably lengthened and the stage of logarithmic growth pushed to a later period. Since determination of the various phases of growth by direct microscopic observation gives completely reliable information concerning the duration and onset of the periods, the growth curve obtained by the plate method may be regarded as an artifact, although this method also shows the well-known phases of growth. I am of the opinion that this can be explained by simultaneous consideration of the growth curves and the morphology of the bacteria. For the first two to three hours the growth on the surface of the agar and in the broth takes place in the same way. In this stage there are no bacteria shadows and the percentage of germination is 100, so that the results of the plate procedure and of direct microscopy are the same. When the cultures get to be 2½ to 3½ hours old, bacteria shadows begin to appear and at the same time the germination capacity falls off. Because of the decreased germination capacity, the curve obtained by the plate method, which should run as a straight line from the second hour on, does not show the true slope. The long latent time that occurs here can be explained by the low resistance in the period of the bacteria shadows. The fact that a stage of logarithmic growth is found in the plate method, although at a later point in time, can be explained, conversely, by the fact that the resistance of the bacteria is again increasing, so that the decreasing rate of fission is compensated for.

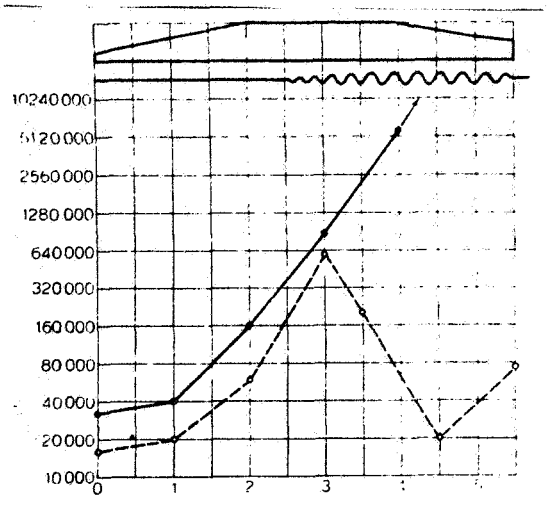
I believe that these experiments justify me in asserting that the growth

phases determined by the plate method are artificial products, inasmuch as they are the result of the interplay of several factors. The difference between the results of bacteria counting and of the plate method, especially in the case of young cultures, is also explained by these experiments. The fact that the curve obtained by the plate method has such a flat pattern and exhibits no downward bend in the period with bacteria shadows (see Experiment 7, where the germinating capacity is less than 10%) may serve as an additional evidence that the shadows are not dead bacteria, but merely bacteria of low resistance. It may therefore be assumed that the method of subdividing on plates, in which the germs are covered on all sides by agar, offers considerably better conditions of growth than inoculation of an agar surface. That something of the same kind also manifests itself in the action of the bacteriophages will be discussed below in some detail.

PART TWO

While my investigations of the occurrence of bacteria shadows were carried out with normal growth of *Bacillus coli*, Oerskov called my attention to the /22 fact that the whole phenomenon is strongly reminiscent of the bacteriophage effect, although in somewhat lesser degree. A search of the relevant literature turned up so many points of correspondence that it was to be feared that the phenomenon mentioned was only a weak bacteriophage effect which could be observed in every culture. Let me briefly state the similarities between the bacteriophage effect and the shadow period. The bacteriophage effect is also tied to a specific period of bacterial growth, in which the bacteria are vigorously subdividing, a period whose time of onset coincides with the shadow period in my experiments.

To demonstrate this, I have compared the experiments in which the bacterial growth was simultaneously measured by direct observation and by the plate method with the experiments of Doerr and Grüninger (*Zeitschrift für Hygiene*, Vol. 97, p. 220, 1923) by plotting the results of the plate method in the two sets of experiments in a single graph, drawing in the number of bacteria shadows in the wavy line at the top (the size of the undulations providing an indication of the number of shadows), and, lastly, indicating the size of the bacteria in the graph (see Curve 12). From these two curves, of which the broken-line curve



Curve 12.

represents the experiments of Doerr and Grüninger, it is immediately evident that the bacteriophage effect and the shadow period coincide, and also that the bacteriolysis dies not with the disappearance of the shadows and the transformation of the culture from large to small growth forms.

This behavior shows up not only in this experiment, but in all those done by Doerr and Grüninger, and Gjörup's experiments exhibit the same pattern.

That this is by no means because the bacteriophage is not concentrated enough until this point of time is quite clear from the experiments of Doerr and Grüninger in which they added the bacteriophage 1) immediately upon inoculation, 2) at a time when the bacteria shadows are just beginning to appear, and 3) when according to my calculations they have disappeared again. In the last case no bacteriolysis set in. (See the experiments in the *Zeitschrift für Hygiene*, Vol. 97, pp. 223-225, 1923.)

As a second similarity, let me point out that the two phenomena are, so to speak, combined surface phenomena after previous smear inoculation. Thus Doerr and Berger [25] and others have found that the bacteriophage effect is suppressed by transplanting in gelatin or agar and does not develop until after smearing on the surface of the agar. I found something similar in my experiments (<10% germination in the shadow period, while the plate procedure used /23 simultaneously showed far greater capacity for germination).

Upon closer consideration, however, it became clear to me that the conditions I had observed in normal bacterial growth could not possibly be due to a bacteriophage effect, but that, nevertheless, there was a certain connection between the two phenomena. I deduced the former proposition from the fact that although a very large number of the *B. coli* strains were investigated under daily reinoculation, no macroscopically observable bacteriolysis has ever been observed either on the surface of the agar or in the broth. The connection between

the two phenomena can be explained by the following argument:

When a bacteriophage is added to a broth that has been inoculated with, e.g., a 24-hour-old culture, the resistance of the bacteria is so great that no bacteriolysis can occur (as has been shown by Doerr and Grüninger and by Gjörup, among others). The bacteria therefore begin to increase in size, and this takes up the latent time and the beginning of the stage of logarithmic growth. Then, because of a shortage of the abovementioned nutrient substance, the weakly structured, low-resistance shadows or their transitional forms begin to appear. The possibility of the onset of bacteriolysis becomes understandable on the basis of this lowered resistance, and especially so after further weakening by smearing on the dry surface of the agar. It is also evident from my germination experiments that even at the peak of the shadow period there are more resistant bacteria present. This helps to explain the fact that bacteriolysis is rarely capable of sterilizing broth completely. If the bacteria later convert to the more resistant, small growth forms, the renewed growth even in cultures with a high lysin concentration becomes explicable (Doerr and Grüninger [23], Gjörup [24]).

Series 7. The Author's Investigations of the Bacteriophage Effect

This series of investigations should not be regarded as a separate little work, a sequel to the preceding investigations, but rather as a completion of those investigations, by which the differences in the bacteria in the various phases of growth and the importance of knowledge of them if one is concerned with investigations in this field are brought out more clearly.

In the numerous articles on d'Herelle's phenomenon, direct microscopic observation of the bacteriophage effect has been used only to a slight extent, and Gjörup, who did use this method, observed only the bacteriolysis itself and the renewal of growth, not the point in time when bacteriolysis sets in or when it ceases. While for the most part Gjörup used an immersion lens, in my experiments I used the same system of lenses as before. The technique was the usual one; i.e., the growth of the same bacterium could be observed from hour to hour on the surface of the agar. The results of these experiments are shown in Tables XII and XIII.

TABLE XII (EXPERIMENT 19). CULTURE: 48-HOUR-OLD AGAR CULTURE. COLI BACTERIOPHAGE: ONE DROP ON THE CUBE OF AGAR

Age of the Culture	1	1½	2½	3	3½	4	4½	5 Hrs.
Colony 1	2÷	2÷	9÷	20÷	36+	17++	3++	0
Colony 2	2÷	2÷	8÷	19÷	44÷	ca.80+	31++	2++
Colony 3	2+÷	2÷	10÷	24÷	46÷	ca.51+	16++	0

÷ = no bacteriophage effect

\+ = weak bacteriophage effect

++ = strong bacteriophage effect

0 = culture dissolved

TABLE XIII (EXPERIMENT 20). CULTURE: 18-HOUR-OLD AGAR CULTURE. COLI BACTERIOPHAGE: ONE DROP ON THE CUBE OF AGAR

Age of the Culture	½	1	1½	2	2½	3	4 Hours
Colony 1	2÷	3÷	6÷	14÷	34+	13++	0
" 2	2÷	2÷	5÷	13÷	33+	16++	0
" 3	1÷	1÷	2+	0	0	0	0
" 4	2÷	3÷	6÷	15÷	41÷	60++	5++
" 5	2÷	4÷	8÷	17÷	44+	56++	5++
" 6	2÷	4÷	8÷	16+	24++	6++	2++
" 7	2÷	4÷	8÷	17÷	39++	8++	0
" 8	2÷	4÷	8÷	17++	4++	0	0
" 9	1÷	2÷	5÷	16÷	48÷	80++	6++
" 10	1÷	2÷	4÷	12+	11++	4+	0
" 11	1÷	1÷	2÷	4++	0	0	0

The meaning of the symbols is the same as in Table XII.

The above experiments confirm the experiments of Doerr and Grüninger [23], /24 for they show that the bacteria apparently multiply normally for the first two or three hours, while the bacteriophage effect sets in quite suddenly at a time when the culture has multiplied at a maximum rate for some time; i.e., has advanced somewhat into the stage of logarithmic growth. It is also evident that a certain time (one to two hours) elapses between the beginning and end of the

bacteriolysis.

If a question arises as to the relationship between bacteriolysis and bacteria shadows, the answer may be given that for this coli strain the shadow phase set in at a rather early point in time, so that there would be nothing in the way of the assumption that the two periods coincide. The objection could be raised against the experiments, however, that even when the bacteriophage is added in fairly high concentration, it is quite impossible to be sure that it is not necessary to have an increase in strength before the lysis can set in. In order to clarify this further and possibly to be able to show that *bacteriolysis sets in only when the culture is in the less resistant phase of growth in which bacteria shadows are in evidence*, the following experiment was performed:

A large agar dish was inoculated with a *B. coli* culture (of the same strain) uniformly and so densely that the growths ran together four or five hours later. Just after inoculation a cube was cut out and a drop of a powerful bacteriophage dropped on it and distributed over the surface *without smearing*. Both the large agar dish and the cube were then put into the constant-temperature cabinet at 37°C, and every hour thereafter a fresh cube of agar was cut out and bacteriophage added to it. All the cubes were subjected to microscopic examination every hour. At the same time the morphology of the primary culture was observed every hour after the smear inoculation. When the culture in the primary agar dish had grown five hours old, a pellicule formed over the whole surface. In order to be able to observe the bacteriophage effect better, we later washed off some of the bacteria of the cube of agar together with the bacteriophage. /26

I believe this cannot introduce any experimental errors, because at that point of time the bacteria were in the process of converting into more resistant forms, which were thus subjected to an extraordinarily strong concentration of bacteriophage. Moreover, this procedure offered the advantage that more or less the same number of bacteria were present on the cubes of agar from the third hour on.

Tables XIV and XV show the results of these experiments in concise form.

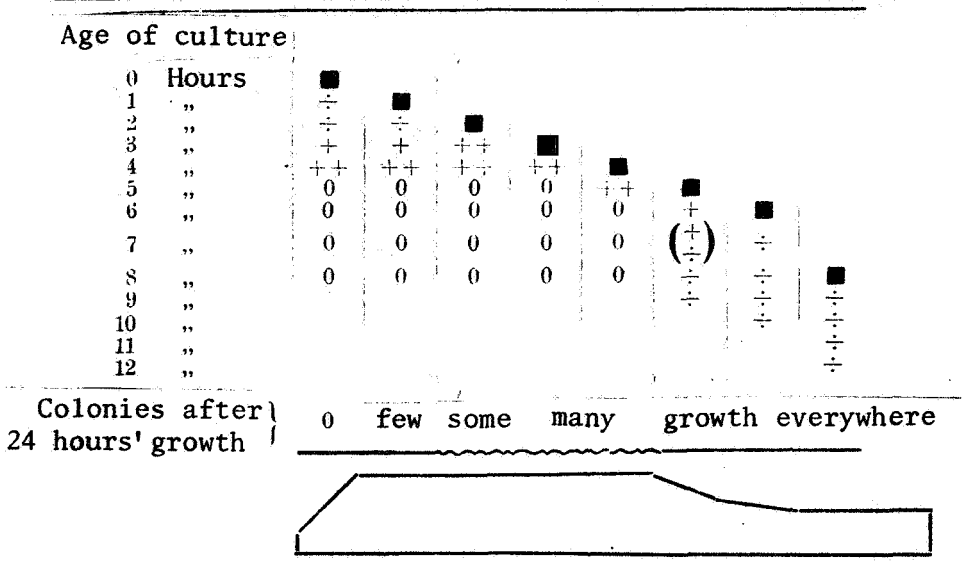
These two experiments, which ran exactly alike in the main, showed the following: If the bacteriophage was added before the period with the bacteria shadows, the growth continued in the normal way, and it was only after the

colonies had reached a certain size (ca. 16 to 100 bacteria), as in Experiments 19 and 20, that the bacteriophage effect began to manifest itself. If the bacteriophage was added during the shadow period, the growth stopped immediately, and only half an hour later a very powerful bacteriophage effect manifested itself. As mentioned in connection with Experiments 19 and 20, however, it is not to be expected that the bacteriolysis will set in instantaneously, even in this period, because the process in itself takes from one to two hours. Lastly, we find that when the bacteriophage is added after the end of the shadow phase and conversion into small growth forms, the bacteriophage effect is completely lacking. The transition between the last two periods is of particular interest. For if the bacteriophage is added to the five-hour-old culture (Experiment 21) or from the fifth to the sixth hour (as in Experiment 22), a fairly strong bacteriolysis sets in in some bacteria, while others continue to multiply and gradually spread over the whole cube of agar. This is explained by the fact that at this stage the cultures consist in part of large growth forms and shadows and in part of small growth forms, and that the first two classes of forms are affected by the bacteriophage, while the last mentioned not only is unaffected, but on the contrary grows over the spots where the bacteria have been dissolved by the bacteriophage.

To judge by these experiments, it seems to me to be justified to draw the conclusion that the bacteriophage effect sets in only when the culture is in the phase of growth that according to my earlier investigations is characterized by the bacteria shadows, in which period the bacteria have very little resistance to harmful influences. This agrees quite well with the investigations of Doerr and Grüninger [23] and Doerr and Berger [25]. It was shown by those investigations that the bacteriophage effect sets in precisely when the culture has multiplied at the maximum rate for some time.

From those investigations and from experiments concerning the antilytic capacity of gelatin, Doerr and Berger [23] drew the conclusion on purely theoretical grounds that bacteriolysis is due to a "dystrophic membrane lesion" of the bacteria. This conclusion seems to be in complete accord with my observations, inasmuch as the lack of the aforementioned nutrient substance or agent /27 produces the bacteria shadows and so brings about the bacteriophage effect.

TABLE XIV (EXPERIMENT 21)

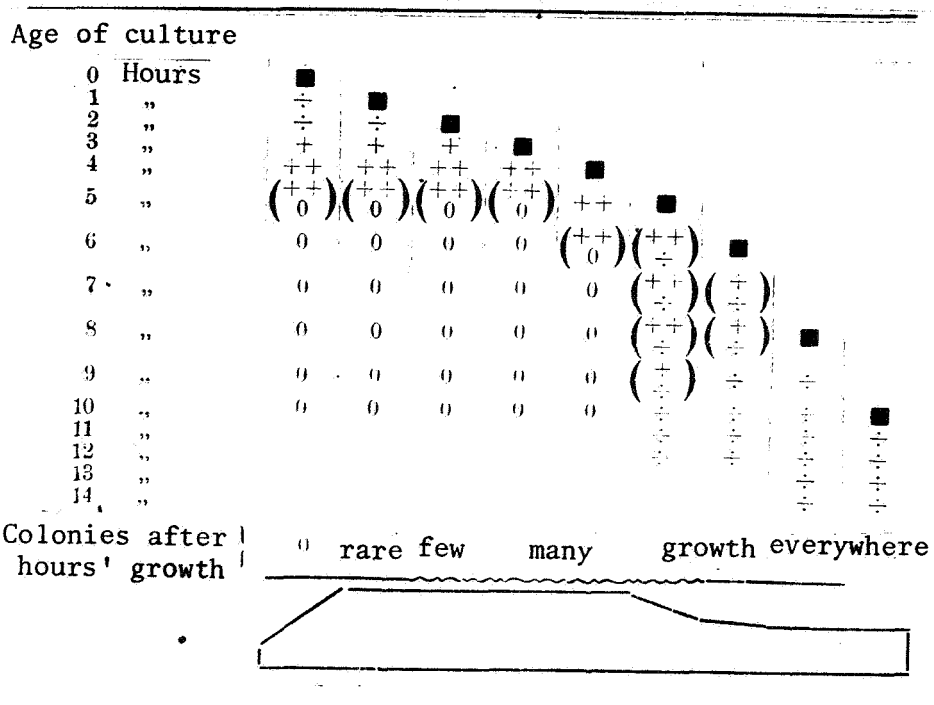


0 = culture dissolved
÷ = no bacteriophage effect
+÷ = bacteriophage effect on some bacteria; simultaneous growth of others
\+ = weak bacteriophage effect
++ = powerful bacteriophage effect

As before, the wavy line at the bottom indicates the phase with the bacteria shadows. The figure at the bottom, as before, indicates the size of the bacteria.

■ indicates the point at which the bacteriophage was added to the culture.

TABLE XV (EXPERIMENT 22)



During my experiments concerning the bacteriophage effect some other observations were made that are to be interpreted in the same direction with those mentioned above. Thus it was found that both the shadows and the bacteriophage effect set in earlier on thin agar plates than on thick ones, and earlier when the bacteria are smeared on the agar more densely than when they are more thinly spread; i.e., in both cases they show up earlier where the aforementioned agent is used up most rapidly. Some experiments in which the cubes of agar with culture and bacteriophage were simultaneously put into the constant-temperature cabinet at 37°C (as in Experiments 21 and 22) and into the humid chamber at 0° showed the well-known phenomenon that the bacteriophage effect was lacking in the latter culture, even when the culture was in a growth stage, while at 37°C bacteriolysis set in almost instantaneously. If after four or five hours at 0° the cubes are again put into the constant temperature cabinet, the bacteriophage effect does not set in within the first hour, as might be expected, but rather after two or three hours. In my opinion this can be explained only on the basis that the abovementioned agent or the nutrient substance, which, as I have already shown, can diffuse through agar, diffuses from the mass of agar at the bottom to the surface during the cold period, and so gives the bacteria the same conditions of growth as they have on a fresh agar surface.

In order to investigate further the relationship between the bacteriophage effect and the substance necessary for rapid bacterial growth, a series of investigations were carried out in which cultures in the substrates described below were examined from hour to hour by direct observation.

The following substrates were prepared in a number of test tubes:

1) 20 cm^3 of filtrate + 1 loopful of bacteriophage
2) 18 cm^3 of filtrate + 2 cm^3 of broth + 1 loopful of bacteriophage
3) 10 cm^3 of filtrate + 10 cm^3 of broth + 1 loopful of bacteriophage
4) 0 cm^3 of filtrate + 20 cm^3 of broth + 1 loopful of bacteriophage.

The filtrate consisted of a 24-hour broth culture filtered through a Chamberland No. 2 filter and brought up to the same pH as the broth. After two days in the constant-temperature cabinet to ensure sterility, the test tubes were inoculated with equal amounts of an 18-hour coli culture and placed in the constant-temperature cabinet. Every hour 2 cm^3 was taken out and centrifuged, the

centrifugate was put on cubes of agar without smearing, so that the morphology of the culture and the bacteriophage effect could be observed. The cubes were then put in the humid chamber at constant temperature and 24 hours later were examined macroscopically.

Table XVI (Experiments 23, 24, and 25) shows only the number of the nutrient medium (see above, page 33); instead of one loopful of the bacteriophage, however, in Experiment 24 two drops were added and in Experiment 25 one cm^3.

TABLE XVI. SUBSTRATES 1, 2, 3, 4 INOCULATED WITH AN 18-HOUR-OLD COLI CULTURE.

		EXPERIMENT 23	
Time in Hours	Sub-strate	Morphology	Bacteriophage Effect
1	1	Few large growth forms	None
	2	Several large growth forms	None
	3	Mainly large growth forms	None
	4	Mainly large growth forms	None
2	1	Some large growth forms, but mainly small bacteria	None
	2	Large growth forms	None
	3	Large growth forms	Suggested
	4	Large growth forms	Suggested
3	1	Some large growth forms, but mainly small bacteria	None
	2	Large growth forms	Fairly powerful
	3	Large growth forms	Very powerful
	4	Large growth forms	Very powerful
4	1	Mainly small bacteria	Suggested
	2	Numerous large growth forms	Very powerful
	3	Few bacteria left	Very powerful
	4	Few bacteria left	Very powerful
5	1	Only small bacteria	None
	2	Few bacteria left	Very powerful
	3	Almost no bacteria left	Very powerful
	4	Almost no bacteria left	Very powerful
6	1	Small bacteria	None
	2	No bacteria	None
	3	No bacteria	None
	4	No bacteria	None

[continued]

TABLE XVI (CONTINUED). EXPERIMENT 24

Time in Hours	Sub-strate	Morphology	Bacteriophage Effect
	1	Some large growth forms, but mainly small bacteria	None
1	2	Mainly large growth forms	None
	3	Mainly large growth forms	None
	4	Only large growth forms	None
	1	Some large growth forms, but mainly small bacteria	Weak
2	2	Large growth forms	Very powerful
	3	Large growth forms	Powerful
	4	Large growth forms	Powerful
	1	Some large growth forms, but mainly small bacteria	Weak
3	2	Large growth forms	Very powerful
	3	Large growth forms	Bacteria have almost completely disappeared
	4	Large growth forms	As above
	1	Mainly small bacteria	Weak
4	2	Only a single bacterium left	Very powerful
	3	Only a single bacterium left	Very powerful
	4	Only a single bacterium left	Very powerful
5 } 6		All substrates unchanged	
	1	Only small bacteria	None
7	2	Only a single bacterium	
	3	Only a single bacterium	
	4	Only a single bacterium	

EXPERIMENT 25

	1	Some large growth forms, but mainly small bacteria	None
2	2	Mainly large growth forms	Weak
	3	All growth forms large	Powerful
	4	All growth forms large	Powerful
	1	Some large growth forms, but mainly small bacteria	Weak
3	2	Large growth forms	Powerful
	3	Large growth forms	Very powerful
	4	Large growth forms	Very powerful

[continued]

TABLE XVI. EXPERIMENT 25 (CONTINUED)

Time in Hours	Sub-strate	Morphology	Bacteriophage Effect
4	1	Some large growth forms, but mainly small bacteria	Weak
	2	Large growth forms	Very powerful
	3	Large growth forms	Very powerful
	4	Few bacteria left	Very powerful
5	1	Mainly small bacteria	None
	2	Large growth forms	Very powerful
	3	Few bacteria left; large growth forms	Some
	4	Only a single bacterium left	Some
6	1	Only small bacteria	None
	2	Large growth forms, only scattered bacteria	Some
	3	Large growth forms, only a single bacterium	
	4	As above	
7		Substrates 1, 2, 3, 4 unchanged.	

EXPERIMENT 24. MACROSCOPIC EXAMINATION AFTER 24 HOURS

1		All cubes sterile.
2		All cubes sterile
3		About four colonies on each cube.
4	1	11 colonies
	2	12 colonies
	3	5 colonies
	4	7 colonies
5	1	50 colonies
	2	45 colonies
	3	35 colonies
	4	46 colonies
6	1	Innumerable colonies
	2	" "
	3	" "
	4	" "

Examination of the cubes of agar in Experiment 24 after 24 hours' growth /30 brought up a new problem. While the cubes of agar dating from the second, third,

and fourth hours and inoculated with the cultures of the four test tubes were almost sterile, the cubes from the fifth, sixth, and seventh hours showed an increasing number of colonies. (Because of the powerful bacteriophage effect, the number of bacteria placed on these cubes was actually less.) This phenomenon seems to me to find its explanation in the occurrence of bacteriophage-resistant bacteria.

Two forms of resistance must thus be distinguished: 1) One due to the fact that the bacteria are small. This resistance disappears when the bacteria develop into large growth forms on a fresh medium. -- 2) A genuine resistance. Here the bacteriophage effect is lacking even after reinoculation on a fresh medium with an abundant addition of bacteriophage.

I shall go into this latter type of resistance somewhat more in detail in the next series of experiments.

Series 8. Investigations of Bacteriophage-Resistant Bacteria

The experiments were carried out partly in broth, partly on agar.

Experiment 26. -- The broth, provided with abundant amounts of bacteriophage, was inoculated with a resistant culture (cultivated twice in broth containing bacteriophage). The morphology of the culture was observed every hour in the manner described earlier.

TABLE XVII (EXPERIMENT 26)

Hour	Morphology	Remarks
2nd	Large growth forms	Suggestion of a bacteriophage effect
3rd	" " "	" " " "
4th	" " "	" " " "
5th	Bacteria decreasing in size	" " " "
6th	Small bacteria	No bacteriophage effect
	Macroscopically After 24 Hours of Growth	
2nd	Colonies growing together	
3rd	" " "	
4th	" " "	
5th	" " "	
6th	" " "	

Experiment 27. -- A large plate of agar was inoculated with a resistant culture. Every hour a cube was cut out, bacteriophages were added, and the morphology was observed each hour.

TABLE XVIII (EXPERIMENT 27)

Cube Taken from Original Culture	Observed After ... Hours	Morphology	Bacteriophage Effect
Immediately	0	Small permanent forms	None
	1	Some large growth forms	None
	2	Large growth forms	A few colonies show powerful effect
	3	" " "	As above
	4	" " "	As above
	5	Bacteria smaller	Some colonies show weak effect
	6	" "	Almost no bacteriophage effect
After 1 Hour	0	Some large growth forms	None
	1	Large growth forms	None
	2	" " "	Some colonies show powerful effect
	3	" " "	As above
	4	" " "	As above
	5	Bacteria smaller	Only insignificant effect
	6	" "	No bacteriophage effect
After 2 Hours	0	Large growth forms	A few colonies show powerful effect
	1	" " "	As above
	2	" " "	As above
	3	" " "	As above
	4	Confluent growth	None
	5	" "	None
After 3 Hours	0	Large growth forms	A few colonies show powerful effect
	1	" " "	As above
	2	Confluent growth	None
	3	" "	None
	4	" "	None
After 4 Hours	0	Large growth forms	None
	1	" " "	Insignificant
	2	Confluent growth	None
	3	" "	None
	4	" "	None

[continued]

TABLE XVIII (EXPERIMENT 27; CONTINUED)

Cube Taken from Original Culture	Observed After ... Hours	Morphology	Bacteriophage Effect
After 5 Hours	0	Confluent Growth	None
	1	" "	None
	2	" "	None
	3	" "	None
	4	" "	None
After 6 Hours	0	Confluent growth	None
	1	" "	None
	2	" "	None
	3	" "	None
	4	" "	None

It is thus clearly shown by these experiments that a genuine resistance is /31 involved, which has come about through interaction between the bacteria and the bacteriophage, and that it is quite different from the resistance mentioned in Point 1, which is due to a quality of the bacteria alone.

The question might now arise whether the resistant culture differs from the normal culture in respect to growth, so that it propagates itself only as small bacteria, or whether the period of growth characterized by the bacteria shadows is lacking, and it is resistant to bacteriophage for that reason.

Accordingly, I carried out cultivation experiments with the resistant culture on agar, measuring the rate of growth and observing the morphology as in Series 4. The growth pattern found here was entirely the same as that of the normal coli culture. It therefore seems to me to be clear that the latter type of resistance is by no means exclusively due to an alteration of the mode of growth of the bacteria themselves, but must be due to another kind of change and one that is induced by the bacteriophage.

Do these experiments offer a possibility of explaining why some strains of *B. coli* are influenced by bacteriophage, while others are uninfluenced by it?

To judge by my experiments dealing with the normal growth of *B. coli*, in /32 which some strains show almost no shadow period, while in others such a period

is very pronounced, it was tempting to assume in advance that the differences in bacteriophage effect were due to this differing behavior of the shadow period. Some bacteriophage experiments which were carried out in part with a coli strain that showed only a brief and not very marked shadow period and in part with a strain in which the shadow period stood out clearly showed, however, that both strains were bacteriophage-resistant, so that this difference in the natural resistance of strains cannot be the primary cause of the bacteriophage effect.

SUMMARY

The present work begins with a brief survey and a criticism of the various methods of quantitative determination of the growth of bacteria. The most important works on the conditions of growth of bacteria are then discussed, and especially Barber's investigations of the growth of *Bacillus coli* in broth.

A technique is then described that is based on Oerskov's pure-culture method and by which it has been found possible to describe and measure bacterial growth by direct microscopic observation.

In this way the growth of *Bacillus coli* on the surface of agar is studied in Series 1 and 2, and the presence of three marked phases of growth is confirmed. These phases, first described by Max Müller, are: 1) *the latent period*, 2) *the phase of logarithmic growth*, and 3) *the period with decreasing rate of growth*.

It was further found that the latent period can be subdivided into two periods: 1) *the absolute latent period*, during which the transplanted bacteria increase in size without undergoing fission, and 2) *the relative latent period*, during which the culture begins to multiply, but has not yet reached the maximal rate of growth.

The individual bacteria multiply at the maximal rate when their absolute latent time is over. The relative latent period is therefore due to the fact that the individual bacteria of the culture exhibit latent periods of varying length. Old cultures show both a longer absolute and a longer relative latent period than young cultures, and also show greater differences in respect to the growth energy of the individuals.

In Series 3 the germinating capacity of several strains of *Bacillus coli*

is investigated and it is found that

a) Quite young cultures exhibit a percentage of germination up to ca. 100%;

b) There is a period in which the germinating capacity undergoes a substantial decrease;

c) After that, a sharp rise in germinating capacity sets in, followed by a /33 period in which the germinating capacity is again nearly 100%, after which it again declines with the age of the culture.

In Series 4 the morphology of the culture is observed and its rate of growth and germinating capacity determined simultaneously; by this means an explanation of the conditions observed in Series 1 to 3 is arrived at.

Immediately after reinoculation of an 18-hour culture, the bacteria show up as small *permanent forms*. In the course of the latent period, these increase in size and are converted into *large growth forms*, all of which are capable of germinating. After the end of the latent period, the culture multiplies uniformly and at a maximal rate during the stage of logarithmic growth. Toward the end of this stage the large, not very refractive *bacteria shadows* appear, which are incapable of germinating under the conditions of growth that are present. The period before the apperance of the shadows corresponds to the *first germination optimum*, while the subsequent period, characterized by the shadows, corresponds to the *period of lowered germinating capacity*.

During this period the number of the bacteria shadows increases, until rather suddenly the culture contains only highly refractive *small growth forms*, all of which are capable of germinating. The *second germination optimum* thus comes about. Since the small growth forms possess less energy for growth than the large ones, the period of logarithmic growth comes to an end at this time.

During the following period the bacteria constantly decrease in size, and their rate of growth also undergoes a decline. In this process they are converted into the *small permanent forms*. Among these, not very refractive forms are again to be found, and as a result the last period sets in, -- the *period of decreasing germinating capacity*.

Series 4 is concluded with two experiments. They show that the growth of

Bacillus coli proceeds in the same way in broth as on the surface of agar.

Series 5 has the objective of finding out the causes of the occurrence of the bacteria shadows. According to these experiments it cannot be assumed that mechanical damage during the reinoculation of the bacteria is to be regarded as the sole cause of the occurrence of the shadows.

It was found that in this period of growth bacteria are to be found in the colonies which are low in protoplasm and not highly refractive, and which can be stained only weakly and only by a special staining technique, and that after they are transplanted by smearing on the surface of agar the aforementioned shadows appear.

Attempts to ascertain the cause of this behavior, namely that the bacteria of a culture, with the shadows as an intermediate member, are converted from large growth forms into small ones, have shown that this is in no way due to metabolic products, but rather to the exhaustion of the nutrient medium with regard to a nutrient substance necessary for rapid growth.

At the same time, it is evident from these experiments that the bacteria /34
shadows are not dead forms, but merely less resistant bacteria.

In Series 6, measurements of the growth of a culture of *B. coli* are made simultaneously by means of the plate method and direct microscopy. It is found that the growth curve obtained by the plate method is an artificial product brought about by the interplay of resistance, storage, and rate of growth.

In a comparison of the observations on bacteria shadows made in this work with the observations of other authors on the bacteriophage effect, a parallelism was found which led to further research in this field.

In Series 7 this relationship is investigated in some detail and it is found as a result that after addition of a bacteriophage to cultures of various ages, the bacteriolysis did not begin until the bacteria shadows appeared, and that after the period of growth characterized by the small growth forms was attained, no bacteriolysis occurred.

It is also demonstrated that the bacteriophage effect is lacking in those cultures in which the bacteria grow as small forms from the outstart (24-hour

filtered broth cultures), but that if 10% of fresh broth is added, the bacteriophage effect sets in almost as quickly and powerfully as in pure broth.

Finally, in Series 8 the question of resistance to bacteriophage is investigated, and two kinds of resistance are found:

1) Genuinely resistant cultures, which remain unaffected by bacteriophage even when they are in the less resistant phase of growth which is characterized by the bacteria shadows.

2) The resistance of the small growth forms and permanent forms, which ceases when they are transplanted to a fresh substrate where they have an opportunity to transform themselves into large growth forms.

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Translated for the National Aeronautics and Space Administration under contract No. NASw-2037 by Techtran Corporation, P.O. Box 729, Glen Burnie, Maryland, 21061.